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Sequence Requirements for Translation Initiation of *Rhopalosiphum padi* Virus ORF2

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Rhopalosiphum padi virus (RhPV) is an aphid-infecting virus with a 10-kb ssRNA genome that contains two large open reading frames (ORFs). ORF1 and ORF2 encode the nonstructural and structural polyproteins, respectively. Both ORFs are preceded by noncoding regions of 500 nt that could function as internal ribosome entry segments (IRESes). The sequence for ORF2 lacks an obvious initiation codon, but an out-of-frame AUG codon is present that could translate ORF2 through a +1 frameshift. To investigate the mechanisms of translation initiation of ORF2, a series of point and deletion mutations were constructed and transcribed and translated *in vitro*. A bicistronic plasmid containing two copies of the RhPV intergenic region translated both ORFs efficiently, indicating that the region functioned as an IRES *in vitro*. Deletion analysis showed that the region required for activity of the IRES extended from 178 nt upstream and 6 nt downstream of the 5′ border of ORF2. Changes in the out-of-frame AUG codon had little effect on translation initiation, but mutations that included the first and second codons of ORF2 or that disrupted a putative pseudoknot structure near the 3′ end of the IRES failed to initiate protein synthesis. Sequence analysis of the *in vitro* synthesized proteins showed that the first amino acid of the polyprotein corresponded to the second codon of ORF2. These results show that *in vitro* the noncoding region upstream of RhPV ORF2 functions as an IRES that contains a pseudoknot that directs translation initiation at a non-AUG codon. If the *in vitro* synthesized proteins have not been processed by an aminopeptidase, translation is initiated at the second (GCA) codon of ORF2.

INTRODUCTION

Nucleotide sequence analysis of insect-infecting RNA viruses has identified two groups of viruses that have amino acid sequence similarity to members of the Picornaviridae. The first group of viruses, which includes infectious flacherie virus (Isawa *et al.,* 1998) and sacbrood virus (Ghosh *et al.,* 1999), has genome organizations similar to those of the Picornaviridae. The viruses in this group each express single polyproteins that contain structural proteins at the amino terminus and nonstructural proteins proximal to the carboxyl terminus. The viruses in the second group, cricket paralysis virus (CrPV; King *et al.,* 1987), *Drosophila* C virus (DCV; Johnson and Christian, 1998), *Plautia stali* intestine virus (PSIV; Sasaki *et al.,* 1998), and *Rhopalosiphum padi* virus (RhPV; Moon *et al.,* 1998), have genome organizations that resemble those of the Caliciviridae. Their genomes contain two large open reading frames (ORFs) with the nonstructural proteins expressed from the 5′ ORF and the structural proteins expressed from the 3′ ORF. These viruses have been grouped into a genus named the Cricket paralysis-like viruses after the first virus of the group to have its RNA at least partially sequenced (Pringle, 1999).

In the RhPV genome, both ORFs are preceded by 500 nt of noncoding RNA, much like 5′ noncoding regions (NCRs) that precede the single ORFs of members of the Picornaviridae (Jackson *et al.,* 1994). The 5′ NCRs of the Picornaviridae facilitate the cap-independent translation of the viral RNAs by functioning as internal ribosome entry sites (IRESes) that allow ribosomes to initiate translation from within rather than from the terminus of the RNA (Jackson *et al.,* 1994). These structures interact with 40S ribosomes and canonical and noncanonical initiation factors to facilitate cap-independent translation initiation. IRESes from different viruses usually have little primary sequence similarity, but they are capable of folding into multilobed and branched structures. There is considerable variation in the size and location of IRESes. Picornavirus IRESes usually are ~450 nt long and can be located as far as 150 nt upstream of the initiation codon. In contrast, the IRESes of members of the Flaviviridae are ~350 nt long and extend ~30 nt downstream of the initiation codon.

Sasaki and Nakashima (1999) recently reported that the intergenic region of PSIV contained an IRES of ~250 nt and that translation of PSIV ORF2 was initiated at a CUU codon. Like PSIV, the RhPV nucleotide sequence does not contain an AUG codon near the beginning of ORF2. However, the RhPV sequence does contain an out-of-frame AUG that could initiate translation of ORF2 through a +1 frameshift. In this study, we examined the

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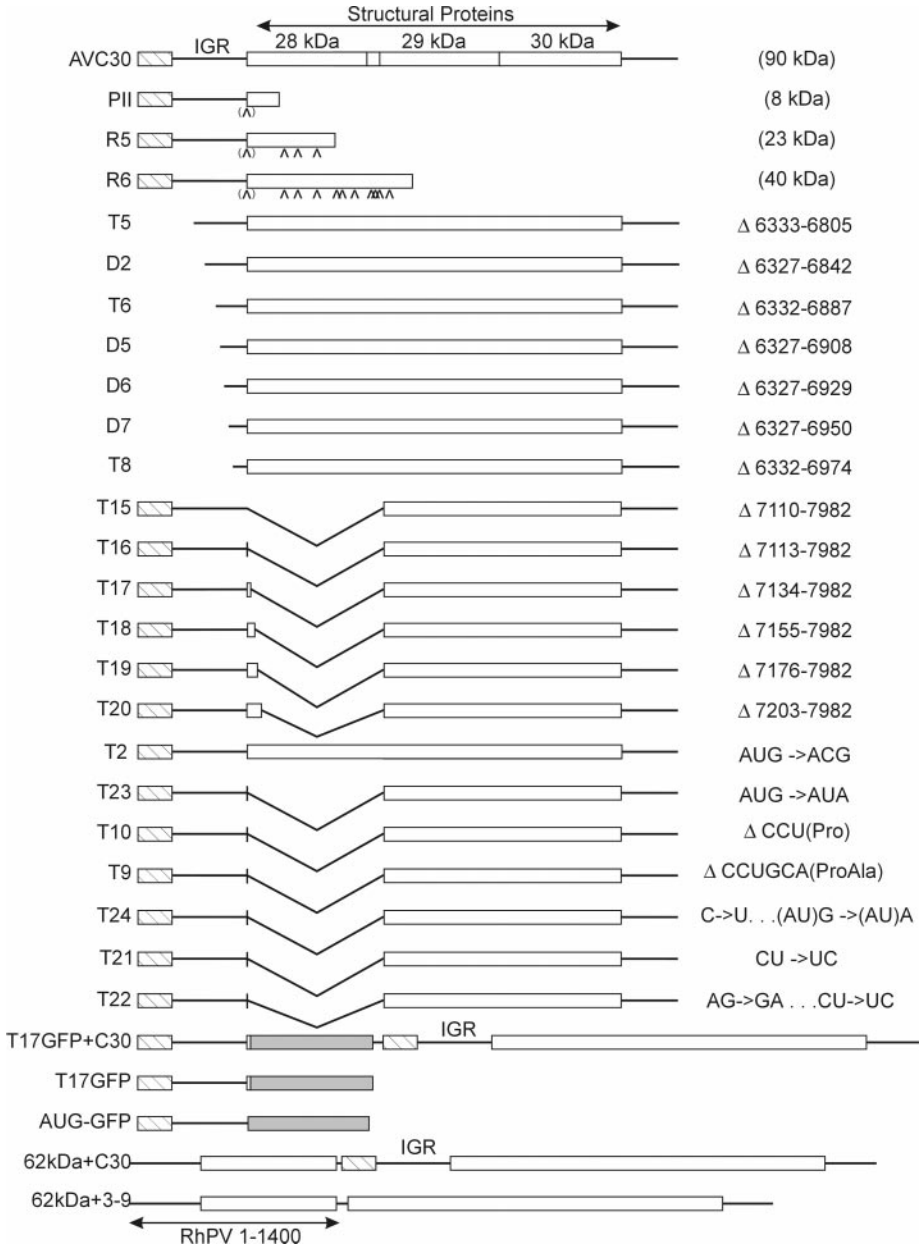

FIG. 1. Clones used to map the boundaries of the RhPV intergenic IRES. Plasmids containing portions of the 3′ end of the RhPV genome were constructed, transcribed, and translated *in vitro*. The run-off transcripts contained a portion of ORF1 (cross-hatched box), intergenic region (IGR), ORF2 (open box) and the 3′-noncoding region. Clones were also constructed where the coding sequence of GFP (shaded box) was positioned downstream of the IGR. For clones PII, R5, and R6, the expected sizes of the proteins are indicated in parentheses and the predicted positions of methionine residues are indicated ( ). The nucleotide changes for the other clones are indicated to the right of each clone.

ability of the intergenic region separating the two RhPV ORFs to function as an IRES and the site of protein synthesis initiation of RhPV ORF2 *in vitro*.

RESULTS

Translation requires high salt

Transcripts from AVC30 (Fig. 1) translated poorly under standard conditions (70 mM KCl, 0.0 mM Mg^{2+}) used for run-off transcripts (Fig. 2A, lane 1). However, at 110 mM KCl and 1.0 mM Mg^{2+}, conditions similar to those used for translation of encephalomyocarditis virus and hepatitis C-like virus (HCV) RNAs (Borman *et al.,* 1995), the RhPV transcript translated well (Fig. 2A, lane 2). Under these conditions, a band of ~90 kDa was observed, which corresponds to the expected mass of a protein translated from near the beginning of ORF2. In addition, protein bands were often seen at ~80 and 70 kDa. To define more closely the translation start site for ORF2, three clones containing 3′ deletions were constructed

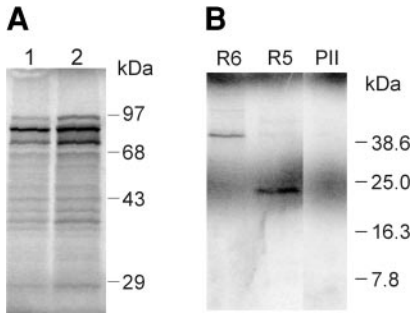

FIG. 2. *In vitro* translation of the RhPV structural proteins. (A) Translation of transcripts from AVC30 in the rabbit reticulocyte lysate in the presence of 70 mM KCl, 0.0 mM Mg^{2+} (lane 1) and 110 mM KCl, 1.0 mM Mg^{2+} (lane 2). (B) Translation products for 3′-deletion clones R6, R5, and PII. No methionine-labeled products were detected from *in vitro* translation with PII, which did not contain an internal methionine residue. The names of the clones are listed above each lane. Unincorporated [^{35}S]methionine comigrated as a dark shadow with the 24-kDa band in the tris-tricine gel system. The migrations of protein molecular mass standards are indicated at the right of each panel.

and transcribed and translated *in vitro*. The masses of two of the protein products (43 and 23 kDa) from deletions R6 and R5 correspond to that predicted from the nucleotide sequence for the initiation of translation at the beginning of ORF2 (40.1 and 23 kDa, respectively; Fig. 2B). No methionine-labeled products were detected from *in vitro* translation reactions with the third deletion clone (PII), which did not contain an internal methionine residue (Fig. 2B).

RhPV intergenic region functions as an IRES

The ability of the RhPV intergenic region to function as an IRES *in vitro* was first tested by inserting the first 1400 nt of the RhPV genome upstream of the insert of pAVC30 (62kDa+C30). However, using the conditions under which ORF2 was translated efficiently, translation products were observed from ORF2 but not from truncated ORF1 (Fig. 3, 62kDa+C30). When the intergenic region upstream of ORF2 was deleted in clone 62kDa+3-9, no translation products were observed from ORF2 (Fig. 3, 62kDa+3-9). To eliminate the dependence on salt concentrations, the mGFP5 sequence was fused in-frame at the ninth codon of ORF2 in clone T17GFP. When this plasmid was transcribed and translated *in vitro*, a protein product corresponding to the expected size of the mGFP5 protein was observed (Fig. 3, T17GFP). When the insert from this plasmid was cloned upstream of the insert of pAVC30 (17GFP+C30) and transcribed and translated, protein bands corresponding to both ORFs were observed (Fig. 3, 17GFP+C30).

Boundaries of RhPV intergenic IRES

The boundaries of the RhPV intergenic IRES were investigated through deletion of sequences upstream and downstream of the 5′ border of ORF2. Transcripts from the clone T5, D2, and T6, which contained deletions of sequences 302, 265, and 220 nt upstream of ORF2, respectively, produced near normal levels of protein products (Fig. 4A). Transcripts from the clone D5 and D6, which contained deletions of sequences 199 and 178 nt upstream of ORF2, respectively, produced reduced but detectable levels of protein products (Fig. 4A). Transcripts from mutants D7 and T8, which contained deletions of 157 and 133 nt upstream of ORF2, respectively, produced barely detectable levels of translation products (Fig. 4A). A set of deletions was constructed from within ORF2 toward the start of the ORF. Mutants T17, T18, T19, and T20 had sequences deleted between nt 7982 and 28, 49, 70, and 97 nt downstream of the start of ORF2, respectively. None of these deletions affected translation initiation (Fig. 4B). Deletion T16, which contained a deletion of sequences 6 nt downstream of the 5′ border of ORF2, showed reduced, but significant, levels of translation (Fig. 4B). This deletion changed the sequence of codons 3 and 4 from AAU AUA to GAC GUC. Transcripts from deletion T15, which contained a deletion of sequences 4 nt downstream of the 5′ border of ORF2, did not show any translation products (Fig. 4B). This deletion changed the sequence of codons 2 and 3 from GCA AAU to GAC GUC. This mutant retained the G at position +4 of ORF2, which is part of the inverted repeat that may form a pseudoknot near the end of the RhPV IRES.

Translation initiation does not use AUG

Previously, we noted the presence of an AUG initiation codon upstream of ORF2 that could initiate translation of ORF2 through a +1 frameshift (Moon *et al.,* 1998). To

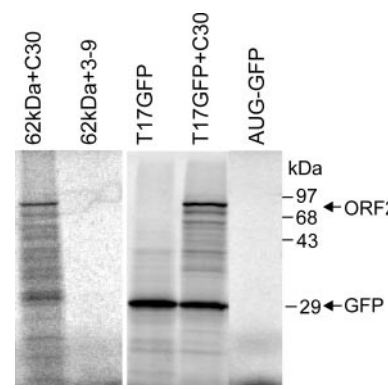

FIG. 3. RhPV intergenic region functions as an IRES. The first 1400 nt of the RhPV genome were cloned upstream of the insert of pAVC30 with (62kDa+C30) and without (62kDa+3–9) the intergenic region. The mGFP5 coding sequence was fused in-frame at the ninth codon of ORF2 in clone T17GFP and in-frame with the AUG codon (AUG-GFP). A bicistronic plasmid was constructed by cloning the insert from T17GFP upstream of the insert of pAVC30 (T17GFP+C30). This plasmid contained RhPV IRES2, mGFP5, a small portion of RhPV ORF1, RhPV IRES2, and RhPV ORF2. The names of the clones are listed above each lane and migrations of protein molecular mass standards are indicated at the right.

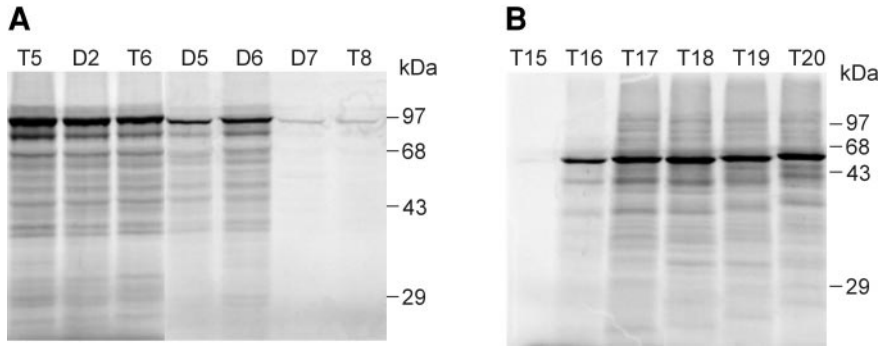

FIG. 4. The 5′ and 3′ boundaries of the RhPV intergenic IRES. Deletion mutants upstream and downstream of the 5′ border of RhPV ORF2 were constructed and transcribed and translated *in vitro*. (A) Transcripts from mutants T5, D2, T6, D5, D6, D7, and T8 contained deletions of sequences greater than 302, 265, 220, 199, 178, 157, and 133 nt upstream of ORF2, respectively. (B) Transcripts from clones mutants T15, T16, T17, T18, T19, and T20 had sequences deleted between nt 7982 and 5, 7, 28, 49, 70, and 97 nt downstream of the start of ORF2, respectively. The names of each deletion are listed above each lane. The migrations of protein molecular mass standards are indicated at the right of each panel.

investigate the possibility that translation of ORF2 is initiated at the out-of-frame AUG, the AUG codon was mutated to ACG (T2) and AUA (T23). The accumulation of protein products from transcripts containing these mutations was reduced but not eliminated (Fig. 5). In contrast, deletion of the first (T10) or first two (T9) codons of ORF2 eliminated the accumulation of protein products (Fig. 5). When the GFP coding region was placed in-frame with the AUG upstream of ORF2, no translation products were observed (Fig. 3, AUG-GFP). When the GFP ORF was positioned in the same reading frame as ORF2 at position +33, which is upstream of the proposed frameshift, the GFP sequence was translated well (Fig. 3, T17GFP).

Translation of RhPV ORF2 requires a pseudoknot

The secondary structure of the RhPV intergenic region predicted by the MFOLD computer program (Mathews *et al.,* 1999) suggests that the region folds into a multilobed structure that contains a pseudoknot at its 3′ border (Fig. 6). To investigate the potential formation of this pseudoknot in initiation of translation of RHPV ORF2, mutations were introduced into one strand of the putative pseudoknot and then complementary mutations introduced that would restore the structure. To complement the G to A mutation present in T23, clone T24 contained a C to U mutation at position 7083, which restored the base pairing at the edge of the pseudoknot structure. Transcripts from T24 restored the translation levels of T23 to near normal levels (Fig. 5). In mutant T21, the putative pseudoknot was disrupted by changing the nucleotides at positions 7109 and 7110 from CU to UC. Transcripts from T21did not produce translation products (Fig. 5). To complement these mutations, clone T22 contained the CU to UC mutations from T21 and complementary AG to GA changes at positions 7079 and 7080, which should restore the pseudoknot structure. Transcripts from T22 translated well (Fig. 5).

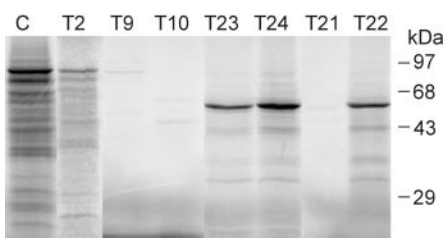

FIG. 5. Translation initiation of RhPV ORF2 does not use AUG and frameshift. The out-of-frame AUG codon at position −4 relative to the start of ORF2 was mutated to ACG (T2) and AUA (T23) and transcribed and translated *in vitro*. Mutants T10 and T9 contained deletions of the first and first and second codons of ORF2, respectively. To test the function of the putative pseudoknot, T23 contained a G-to-A mutation position 7105. T24 contained the G-to-A change from T23 and a complementary C-to-T mutation at position 7083. T21 contained CT-to-TC changes at positions 7108 and 7109 in the middle of the pseudoknot inverted repeat. T22 contained the CT-to-TC mutations from T21 and complementary AG-to-GA changes at positions 7079 and 7080. A wild-type positive control (C) was included in each reaction. The names of each clone are listed above each lane. The migrations of protein molecular mass standards are indicated at the right.

Identification of the site of translation initiation

Attempts to determine the translational start site of the *in vivo* synthesized ORF2 polyproteins were not successful, because the amino terminus of the 28-kDa capsid protein was blocked (Moon *et al.,* 1998). As an alternative, the translational start site used *in vitro* was determined by translating the transcripts from pAVC30 in the presence of [^{3}H]isoleucine. If translation is initiated at the first codon of ORF2, an isoleucine residue would have been expected in cycle four. However, a labeled isoleucine residue was seen in cycle three (Fig. 7).

DISCUSSION

In these studies, we demonstrated that the intergenic region preceding ORF2 of RhPV functions *in vitro* as an IRES that contains a pseudoknot at its 3′ border. The

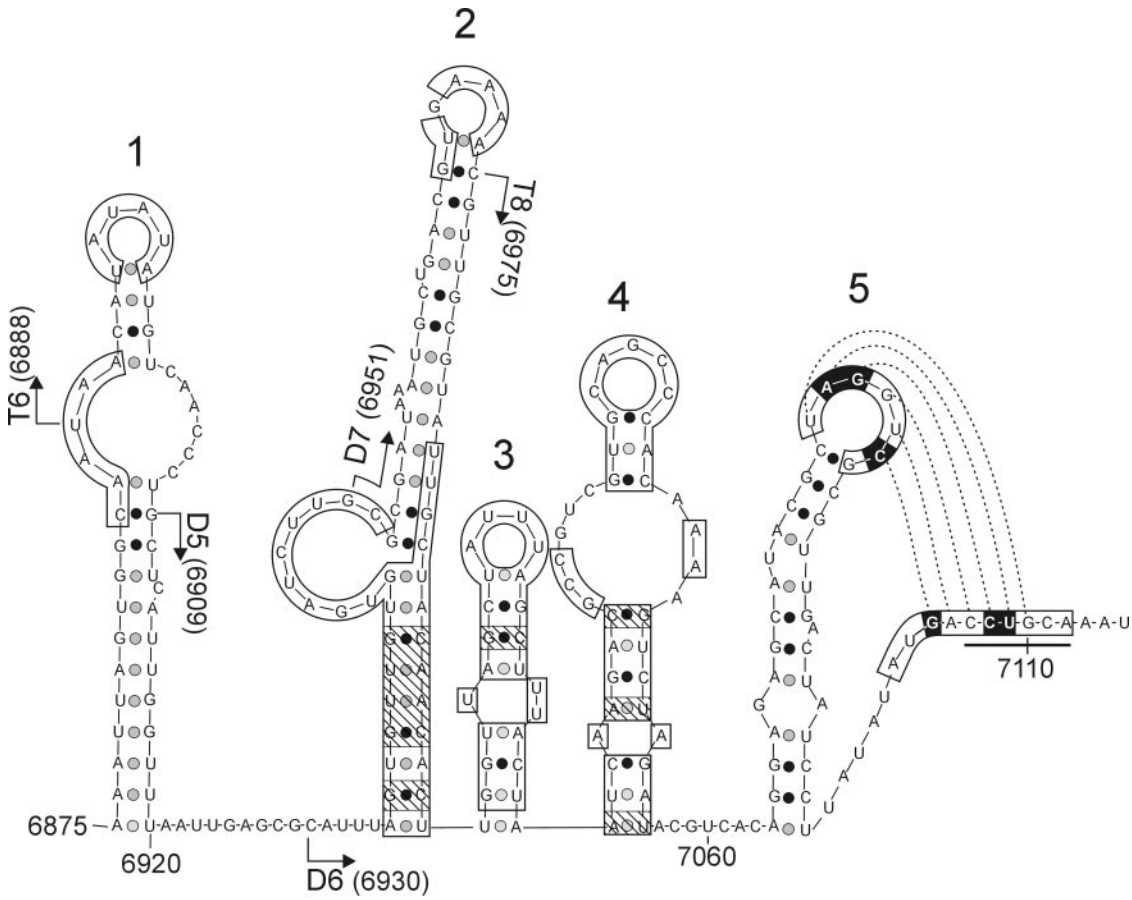

FIG. 6. Predicted secondary structure of the IRES region of RhPV. The 5′ termini of deletions upstream of ORF2 are indicated with arrows. Identical nucleotides in the RhPV and DCV sequences are boxed. Nucleotide positions that are not identical, but preserved secondary structures are indicated by shaded boxes. The base pairing in the predicted pseudoknot is indicated by lines connecting the paired nucleotides. The first two codons of ORF2 are underlined. The positions of mutations in the inverted repeat of the putative pseudoknot structure are indicated as white letters on a black background.

minimal sequence required for translation initiation extended from 178 nt upstream and 6 nt downstream of the 5′ border of ORF2. Previously, we speculated that translation of ORF2 may have begun with an out-of-frame AUG codon and then continued via a +1 frameshift (Moon *et al.,* 1998). However, we found no support for this hypothesis. Mutations in the AUG codon upstream of ORF2 reduced translation, but mutations in the first and second codons of ORF2 abolished translation initiation. Similarly the mGFP5 coding sequence was expressed efficiently when it was inserted into the same reading frame as ORF2, but no expression was seen when mGFP5 was placed in-frame with the AUG codon. These data suggest that translation of RhPV ORF2 is initiated at a non-AUG codon *in vitro*. These findings are similar to those reported recently for another insect-infecting RNA virus, PSIV (Sasaki and Nakashima, 1999).

Mutation and amino acid sequence data suggest that translation of RhPV ORF2 is initiated either at the first (CCU) codon of ORF2 followed by catalytic removal of the amino-terminal methionine or, directly, at the second codon of ORF2 (GCA) through a mechanism that does not involve the use of a methionyl-tRNA. Sasaki and Nakashima (1999) proposed that translation of PSIV ORF2 begins at a position that corresponds to the first codon of the RhPV ORF2 sequences and that the penultimate amino acid is removed by an aminopeptidase. This proposal is consistent with the results presented here where the amino terminus of the *in vitro* synthesized protein corresponds to the second codon of ORF2 (Fig. 7), but lacks a methionine (Fig. 2B). Both the CCU and GCA codons of RhPV ORF2 were required for translation initiation. The first four nt of ORF2 are part of an inverted repeat that mutation analyses have shown to be capable of forming a pseudoknot with the loop at the top of stem 5 (Fig. 6). This inverted repeat is common to all Cricket paralysis-like viruses. For this reason, we hypothesize that the CCU codon is required as part of the pseudoknot to position 40S ribosomes for translation, much like members of the Flaviviridae (Rijnbrand *et al.,* 1997; Pestova *et al.,* 1998).

The IRESes of members of the Picornaviridae and Flaviviridae have been studied in detail. They range in size from ~450 to 350 nt. At 186 nt (RhPV) and 250 nt

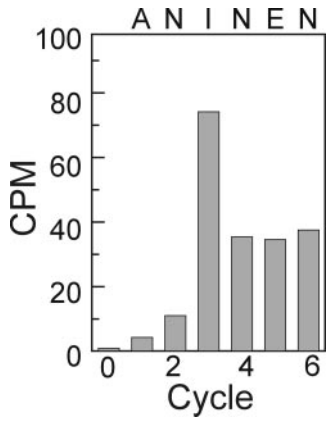

FIG. 7. Sequence analysis of *in vitro* synthesized proteins. Transcripts from pT17 were translated in the presence of [^{3}H]isoleucine. The proteins were subjected to Edman degradation reactions and the radioactivity of cleavage products was determined by liquid scintillation counting. Cycle 0 shows the amount of [^{3}H] liberated before the first cleavage reaction. The predicted amino acid sequence of the beginning of ORF2 is indicated at the top of the graph.

(PSIV), the IRESes of the Cricket paralysis-like viruses are much smaller than those of other previously characterized viruses. Picornavirus IRESes have been divided into three groups. The 3′ boundaries of Type I IRESes (entero- and rhinoviruses) are located ~150 nt upstream of the AUG codon, and initiation codon selection occurs by ribosome scanning (Belsham and Sonenberg, 1996). The activity of Type I IRESes also is very sensitive to salt concentration and is enhanced by virus-encoded proteases (Borman *et al.,* 1995). The 3′ boundaries of Type II and III IRESes (cardioviruses and aphthoviruses and hepatitis A virus, respectively) are located adjacent to the initiation codon and codon selection involves little or no ribosome scanning. The activities of Type II and III IRESes are much less sensitive to salt concentration, and their activity is not enhanced by the addition of picornaviral proteases. The IRES of HCV, a member of the Flaviviridae*,* has *in vitro* translation characteristics similar to those of the picornavirus Type II IRESes, but the 3′ boundary of its IRES is located ~30 nt downstream of the initiation codon. Translation from the HCV IRES does not involve ribosome scanning. Rather the site of translation initiation is determined in large part by a pseudoknot near the 3′ boundary of the IRES (Wang *et al.,* 1995; Pestova *et al.,* 1998). Similarly, the 3′ border of RhPV intergenic IRES corresponds to the first two codons of ORF2 and contains a pseudoknot at its 3′ border.

The predicted secondary structure of the RhPV intergenic IRES contains five prominent stem-and-loop structures and a pseudoknot at its 3′ border (Fig. 6). This structure is very similar to that proposed for PSIV (Sasaki and Nakashima, 1999). Stem-and-loop structures 2–4 are the most highly conserved among the Cricket paralysis-like viruses. All five stem-and-loop structures were required for full activity, but mutants that contained only stem and loops 2–5 translated moderately well. There are several base substitutions among the three sequences that maintain these stem-and-loop structures (Fig. 6). Other short, conserved sequences are found in regions predicted to be single stranded where they could interact with factors involved in translation initiation. Stem and loop structures 2–4 also contain sequences that resemble motifs contained within aphthovirus IRESes that are required for internal initiation of translation (de Quinto and Martinez-Salas, 1997). The features include the GNRA motif at the top of stem 2, the RAAA motif in a loop on stem 4, and a sequence (GCCC) in a loop on stem 4 that is similar to the ACCC motif. These sequences are conserved in all three CrPV-like viruses and may represent the site of ribosome entry for these viruses. Outside this region, there is little primary sequence similarity with the IRESes of other viruses.

The *in vitro* translation characteristics of the RhPV IRES and the presence of a 3′ pseudoknot are similar to the properties of some members of the Flaviviridae, including HCV, hog cholera virus, bovine diarrhea virus, and classical swine fever virus (CSFV) RNAs (Wang *et al.,* 1995; Rijnbrand *et al.,* 1997). Studies suggest that the IRESes and pseudoknots of the Flaviviridae function together to recruit 40S ribosomes to the site of translation initiation in the absence of translation factors, eIF-4E and eIF-4F, which are involved in cap-dependent translation initiation. These structures also seem to position ribosomes at the site of protein translation initiation with little or no subsequent scanning (Reynolds *et al.,* 1995; Rijnbrand *et al.,* 1997; Pestova *et al.,* 1998). While translation of the CSFV polyprotein usually begins at an AUG codon, it also will direct initiation in a positionally dependent manner at other non-AUG codons, including AUU, CUG, and ACG (Reynolds *et al.,* 1995). The data presented here suggest that Cricket paralysis-like viruses may use a similar translation mechanism that involves a pseudoknot structure to position 43S particles for the initiation of translation of their structural proteins. In this mechanism, initiation of translation would be positionally constrained by binding of initiation factors to the IRES, much like prokaryotic translation initiation, where the 30S ribosomal subunit binds to the Shine-Delgarno sequence during translation initiation (McCarthy and Brimacombe, 1994).

During ribosome scanning, the sequence surrounding the initiation codon and secondary structures downstream have been shown to play a role in initiation at non-AUG codons (Kozak, 1997). The presence of a G in position +4 also enhances translation initiation at both AUG and non-AUG codons (Kozak, 1997). The deletion clone T16, which retained just the first 6 nt (CCU GCA) of ORF2, translated well. These data suggest that downstream secondary structures do not play a significant role in codon selection *in vitro*. If translation initiation of

RhPV ORF2 occurs at the CCU codon, the nucleotide at +4 is a G. In the deletion clone T15 the first 6 nt of ORF2 were changed from CCU GCA to CCU GAC. Even though T15 retained the inverted repeat that forms the pseudoknot and a G residue at +4, it failed to initiate translation, which indicates that the primary sequence of the second codon also is an important determinant of codon selection.

The data presented here and by Sasaki and Nakashima (1999) show that the Cricket paralysis-like viruses possess unique properties that facilitate the direct expression of polycistronic RNAs. The intergenic regions of both RhPV and PSIV contain IRESes with features that resemble those of the apthoviruses and flaviviruses. In addition, both initiate translation of their structural protein ORFs at non-AUG codons that are located at the 3′ boundaries of relatively short IRESes. In future studies, we plan to examine the interaction of the RhPV intergenic region with initiation factors to understand the mechanisms by which RhPV initiates protein synthesis of ORF2.

MATERIALS AND METHODS

Production of mutants

The cDNA clone AVC-30 was synthesized from RhPV RNA as described previously (Moon *et al.,* 1998). Deletions upstream of the RhPV intergenic IRES were constructed by inverse polymerase chain reaction (PCR) by the method of Weiner and Costa (1995) using a primer at position 6333 with primers at positions 6805, 6842, 6887, 6908, 6929, 6950, and 6974. Downstream deletions were constructed using a primer at position 7982 with primers at positions 7110, 7113, 7134, 7155, 7176, and 7203. These primers contained *Aat*II sites at their termini. The PCR products were cleaved with *Aat*II, ligated, and used to transform *Escherichia coli* (Sambrook *et al.,* 1989). To determine the reading frame used for expression of the coat proteins, the coding region for mGFP5 (Haseloff *et al.,* 1997) was substituted for that of the capsid proteins. Mutant AUG-GFP contained the mGFP5 sequence fused to the AUG codon in the −4 position relative to ORF2; mutant T17GFP contained the mGFP5 sequence in-frame with the ninth codon of ORF2. To determine the region containing the translational start site for ORF2, three 3′-terminal deletions were made in ORF2. PII was constructed by linearizing AVC-30 with *Pvu*II at position 7321. R5 and R6 were constructed by amplifying pAVC30 with a primer upstream of the T7 promoter and primers at positions 7729 and 8192, respectively. The PCR products were transcribed directly as described below. To investigate the codon used for translation initiation, the AUG codon was changed to ACG (T2) and AUA (T23). Two additional deletion mutants were constructed that deleted the first codon (CCU; T10) and the first and second codons (CCU GCA; T9). To test the function of the putative pseudoknot in translation initiation, a second-site reversion mutant, T24, was constructed for T23 by changing the C at position 7083 to T. Also, mutant T21 was constructed by changing nucleotides at positions 7108 and 7109 from CT to TC, which disrupted the putative pseudoknot. A second-site reversion mutant, T22, was constructed from T21, which changed nucleotides at positions 7079 and 7080 from AG to GA and restored the pseudoknot. Mutants T21, T22, T23, and T24 also contained deletions from position 7116 through 7982. Finally, a bicistronic plasmid containing two copies of the RhPV intergenic region was constructed by inserting either the first 1400 nt of the RhPV genome or the insert of T17-GFP upstream of the intergenic region of pAV-30. The sequences of all mutants were verified by dideoxy chain termination sequencing (Sanger *et al.,*1977) using an Applied Biosystems model 377 automated sequencer.

In vitro transcription and translation

Plasmids were linearized with *Kpn*I, made blunt-ended with the Klenow fragment of *E. coli* DNA polymerase I (Sambrook *et al.,* 1989) and transcribed with T7 RNA polymerase (Promega) according to the manufacturer's protocols. *In vitro* translation reactions (25 μl) using the rabbit reticulocyte lysate were conducted according to the manufacturer's (Promega) protocols. Translation reactions programmed with transcripts containing the RhPV intergenic region were adjusted to 1.0 mM magnesium acetate, 110 mM KCl. Translation reactions programmed with other transcripts were performed at 70 mM KCl without the addition of magnesium acetate. Aliquots of each translation reaction were separated on either sodium dodecyl sulfate (Laemmli, 1970) or tricine-sodium dodecyl sulfate (Schägger and von Jagow, 1987) polyacrylamide gels. The gels were dried and imaged with a Molecular Dynamics Phosphoimager for 18–24 h.

Protein sequence analysis

The amino terminus of the ORF2 polyproteins synthesized from pT17 transcripts in the rabbit reticulocyte lysate was determined by performing the *in vitro* translation reactions in the presence of [^{3}H]isoleucine. In addition, the reactions were supplemented with 0.02 mM of each of the remaining unlabeled 19 amino acids. Proteins were separated by SDS–polyacrylamide gel electrophoresis as above and blotted to ProBlot PVDF membranes (BioRad) as described by Moos *et al.,* (1988). The filters were aligned with prints from the phosphoimager and regions corresponding to the labeled 68-kDa proteins were excised. Proteins bound to the filters were subjected to Edman degradation reactions using an Applied Biosystems model 477A automated sequencer. Individual products of each cycle were dried and counted in a Beckman 2000 scintillation counter.

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